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**A NOVEL FUNGAL PROTEIN CRITICAL FOR
EXPRESSION OF FUNGAL PROTEINS**

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This application claims priority under 35 U.S.C. § 119 from provisional patent application Serial No. 60/074,100, filed February 9, 1998, the entire disclosure of which is incorporated by reference herein in its entirety.

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Field of the Invention

This invention pertains to proteins required for activated transcription in yeast and fungi, nucleic acids encoding these proteins, and methods of using these proteins.

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Background of the Invention

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Most fungi are opportunistic pathogens, producing serious disease only in compromised individuals. As the result of an aging population and an increase in the number of immunocompromised patients, specifically patients with acquired immunodeficiency syndrome (AIDS), patients undergoing cancer and corticosteroid therapy, as well as in patients undergoing organ transplantation, fungal infections are increasing rapidly.

Most infections begin by colonization of either the skin, a mucosal membrane, or the respiratory epithelium. Passage through the initial surface barrier is accomplished through a mechanical break in the epithelium or enzymatic degradation

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- 5 or spore dissemination. Most fungi are readily killed by neutrophils and are only opportunists, but some species are resistant to phagocytic killing and may infect otherwise healthy individuals.

Fungi parasitize many different tissues. Superficial fungi cause indolent lesions of the skin. Subcutaneous pathogens cause infection through the skin and spread by subcutaneous or lymphatic routes. Opportunistic fungi such as *Aspergillus* are widespread in the environment and are present in normal flora and cause disease mostly in immunocompromised individuals. Systemic fungi are the most virulent and may cause progressive disease leading to deep seated visceral infections in otherwise healthy individuals (see e.g. *Sherris Medical Microbiology*, 10 *Third Edition*, Kenneth J. Ryan, ed., Appleton & Lange, Norwalk, CT, 1994).

The major fungal pathogens in North America are *Histoplasma capsulatum*, *Coccidioides immitis*, *Blastomyces dermatitides*, *Cryptococcus neoformans*, *Candida* species and *Aspergillus* species (*Medically Important Fungi*, 15 *Second Edition*, Davise H. Larone, Ed., American Society for Microbiology, Washington, D.C.). *Histoplasma capsulatum* causes histoplasmosis, which may be chronic or progressive and fatal. It is either a localized or disseminated infection, primarily of the reticuloendothelial system. *Coccidioides immitis* causes coccidioidomycosis, a highly infectious disease that is endemic to the southwestern United States and may be a chronic, sometimes fatal infection involving the skin, 20 bone, joints, lymph nodes, adrenal glands and central nervous system. *Blastomyces dermatitides* causes blastomycosis, a chronic infection characterized by suppurative and granulomatous lesions that begins in the lungs and is disseminated to the skin and bones. *Cryptococcus neoformans* causes cryptococcoses, which may be a chronic infection involving the central nervous system. *Candida albicans* is the most frequent 25 cause of candidiasis, which ranges from an acute to a chronic infection involving any part of the body. *Aspergillus fumigatus* is one of the most frequent causes of aspergillosis, which is an opportunistic infection in immunosuppressed individuals. 30

Fungi are a distinct class of microorganism, most of which are free-

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- 5 living. They are eukaryotic organisms containing a nuclear membrane, mitochondria and an endoplasmic reticulum. The cell structure includes a rigid cell wall of mannan, glucan, and chitin and a cytoplasmic membrane with a large percentage of ergosterol. The size and morphology of fungi vary. There are monomorphic yeasts and yeast-like organisms including *Candida*, *Cryptococcus*, and *Saccharomyces*. There are
- 10 monomorphic molds, such as *Aspergillus* and *Coccidioides*. Some thermally dimorphic fungi, such as *Blastomyces dermatitides* and *Histoplasma capsulatum*, grow either in a yeast or mold phase.

- Only a handful of agents are active against fungi. For life threatening disease caused by any of these fungi, amphotericin B is the agent of choice. This
- 15 drug, however, is associated with numerous severe side effects such as fever, dyspnea and tachycardia, and dosage is limited over the lifetime of the patient because of renal toxicity. An agent frequently used concurrently is flucytosine, a nucleoside analog that cannot be used independent of other agents because of the rapid appearance of resistance. Untoward effects of treatment with flucytosine include leukopenia,
- 20 thrombocytopenia, rash, nausea, vomiting, diarrhea, and severe enterocolitis.

- In conditions where the patient's life is not threatened, ketoconazole can be used as a long-term therapy for blastomycosis, histoplasmosis, or coccidioidomycosis. Fluconazole also has a significant role in the treatment of superficial fungal infections. Both compounds are from the same class, the triazoles,
- 25 and are cytostatic. The emergence of resistance and hepatic toxicity limit the use of triazoles such as fluconazole and ketoconazole. The newest triazole, itraconazole, has similar pharmacokinetics and spectrum of activity as fluconazole. None of the azoles can be used for life threatening or deep seated fungal infections, but they are effective in reducing colonization of fungi such as *Candida* and for treating superficial
- 30 mycoses.

All major antifungal agents attack directly or indirectly a component of the cell wall--ergosterol. Amphotericin B and other polyene macrolides interact with ergosterol in the cell membrane and form pores or channels that increase the

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5 permeability of the membrane. Resistant to amphotericin B in mutant strains is accompanied by decreased concentrations of ergosterol in their cell membranes. Imidazoles and triazoles inhibit sterol 14- α -demethylase, a microsomal cytochrome P₄₅₀-dependent enzyme system. Imidazoles and triazoles thus impair the biosynthesis of ergosterol for the cytoplasmic membrane and lead to the accumulation of 14- α -
10 methyl sterols, which impair certain membrane-bound enzyme systems (See e.g. *The Pharmacological Basis of Therapeutics, Eighth Edition*, Goodman and Gilman, Pergamon Press, 1990).

Development of an effective method and composition for treatment of fungal infections is a critical goal of the pharmaceutical industry. The pharmaceutical
15 industry has made numerous efforts to identify fungal-specific drugs, with only limited success to date. It would be of great value to identify a new class of antifungal drug that blocks a fungal target other than ergosterol. This target should be fungal-specific and should lead to development of a drug that is effective against the organisms that are resistant to current therapy.

20 Drug development often relies on the screening of a large number of potential inhibitors before a specific lead compound inhibitor is found. Assays developed for such screens are complex and must mimic the physiological activity of the target protein. Thus, it is critical for the development of these screens to define the proteins involved in the targeted process and to have discovered a means of purifying
25 the necessary components of the assay for use in the assay. In addition, it is useful to have clones for the protein components of the assay to facilitate the production of the components.

Therefore, there is a need in the art to identify one or more fungal constituents, preferably polypeptides, that can serve as useful targets for drug
30 intervention, and for methods and compositions for identifying useful anti-fungal agents and treating fungal infections.

5 Summary of the Invention

The present invention provides an isolated fungal polypeptide, termed TAF-145, that is necessary for activated transcription of particular genes (i.e., gene-specific transcription) in *Candida albicans*. The invention also includes nucleic acid sequences encoding TAF-145, as well as DNA vectors and transformed cells suitable for recombinant expression of this polypeptide. The DNA sequence of *C. albicans* TAF-145 (SEQ ID NO:1) is set forth in Figures 3A-3G.

In one aspect, the present invention provides methods and compositions for inhibiting gene-specific transcription in *C. albicans*, comprising contacting the cell with an agent that selectively interferes with the transcriptional activation activity of the *Candida* TAF complex, preferably with the activity of TAF-145. In one embodiment, the inhibitory agent is a fragment of TAF-145 that inhibits TBP-TAF interaction.

In another aspect, the invention provides a method for high-throughput screening of large numbers of test compounds to identify an agent useful in the treatment of fungal diseases, specifically those caused by *C. albicans*. The method is carried out by exposing the TAF complex or TAF-145 to TATA Box Binding Protein (TBP) in the presence of at least one test compound, followed by determining that the compound inhibits the binding of TBP to the TAF complex or TAF-145. In another embodiment, candidate antifungal agents are identified as those that bind directly to TAF-145, which are identified using methods such as, e.g., those disclosed in U.S. Patent 5,585,277. In yet another embodiment, candidate antifungal agents are identified as those that inhibit the histone acetyl transferase (HAT) activity of TAF-145.

These and other aspects of the present invention will be apparent to those of ordinary skill in the art in light of the present description, claims and drawings.

5 **Brief Description of the Drawings**

Figures 1A-1C together illustrate a protein sequence comparison among TAF-145 derived from *Saccharomyces cerevisiae* (SEQ ID NO:3), *S. pombe* (SEQ ID NO:4), *Drosophila* (SEQ ID NO:5), and humans (SEQ ID NO:6).

Figure 2A is a schematic illustration of the cloning strategy used to
10 obtain the sequence of *C. albicans* TAF-145 (SEQ ID NO:1) using degenerate oligonucleotide PCR.

Figure 2B is a photographic illustration of an agarose gel in which TAF-145-specific PCR products are displayed.

Figures 3A-3G together depict an illustration of the entire nucleotide
15 sequence of the *C. albicans* TAF-145 gene (SEQ ID NO:1) and the predicted amino acid sequence (SEQ ID NO:2).

Figures 4A-4D together illustrate a protein sequence comparison between *C. albicans* (SEQ ID NO:2) and *S. cerevisiae* (SEQ ID NO:3) TAF-145.

Figures 5A-5C together illustrate a protein sequence comparison
20 among the TAF-145 proteins from three yeast species: *C. albicans* (SEQ ID NO:2), *S. cerevisiae* (SEQ ID NO:3), and *S. pombe* (SEQ ID NO:4).

Figures 6A-6C together illustrate a sequence comparison among the TAF-145 homologs from *C. albicans* (SEQ ID NO:2), *S. cerevisiae* (SEQ ID NO:3), *S. pombe* (SEQ ID NO:4), *Drosophila* (SEQ ID NO:5), and human (SEQ ID NO:6).

Figure 7A is an photographic illustration of the results of an
25 experiment in which a *S. cerevisiae* strain temperature sensitive for TAF-145 was transformed with DNA encoding either *C. albicans* TAF-145 or *S. cerevisiae* TAF-145. Only the *Saccharomyces*-derived sequence was able to support growth of the strains under restrictive conditions.

Figure 7B is an photographic illustration of the results of an experi-
30 ment in which a *S. cerevisiae* strain deleted for TAF-145 was transformed with DNA encoding *C. albicans* TAF-145 or *S. cerevisiae* TAF-145. Only the *Saccharomyces*-derived sequence was able to support growth of strains under restrictive conditions.

5 Figures 8A and 8B together illustrate a sequence comparison among the histone acetyltransferase (HAT) domains from *C. albicans* (SEQ ID NO:7), *S. cerevisiae* (SEQ ID NO:8), *S. pombe* (SEQ ID NO:9) and human (SEQ ID NO:10).

 Figure 9 is a photographic illustration of an SDS-PAGE gel of extracts from uninduced (U) and induced (I) cells following Ni-NTA chromatography.

10 Figure 10A is an illustration describing the electroelution of TAF proteins from an SDS-PAGE gel.

 Figure 10B is a photographic illustration of the purity of the electroeluted proteins as determined by a coomassie stained 12 % SDS-PAGE gel.

15 Figure 11 is a graph depicting the results of the ELISA for testing the specificity of rabbit sera against the recombinant CaTAF145 HAT domain.

 Figure 12 is a photographic illustration of a Coomassie stained 12% SDS-PAGE gel and Western blot analysis to examine the expression of recombinant TAF protein in Baculovirus.

20 Figure 13A is a schematic illustration of the strategy used in the deletion analysis of *C. albicans* TAF145.

 Figure 13B is a photographic illustration of the Southern blot analysis to determine if the deletion was successful.

Detailed Description of the Invention

25 All patent applications, patents, and literature references cited in this specification are hereby incorporated by reference in their entirety. In case of conflict, the present description, including definitions, will control.

Definitions:

30 "Basal transcription" refers to transcription activity from an RNA polymerase II-directed promoter in the absence of an upstream transcriptional activator.

 "Coactivator activity" refers to the activity that allows an upstream transcription factor such as GAL4 or its derivatives to activate transcription from an

- 5 RNA polymerase II-directed promoter in an *in vitro* or *in vivo* reconstituted transcription system. Coactivator activity is further defined as an activity that has no effect on basal transcription.

"TATA-box binding protein" or "TBP" is a major component of eukaryotic transcription factors. In fungi and in higher eukaryotes, TBP is isolated as
10 part of a larger protein complex.

"TATA-box binding protein- associated factors" or "TAFs" as used herein refers to polypeptides or complexes of polypeptides required for "coactivator activity" in fungal RNA polymerase II transcription reactions by virtue of their association with TBP.

- 15 "Functional homology" between TAF polypeptides or complexes of polypeptides indicates that one or more biochemical properties specific to fungal TAFs are shared. Examples of such properties are: the ability to specifically modulate the transcription from RNA polymerase II-directed promoters in the presence of an upstream activator protein, the capacity to specifically bind TBP as a multisubunit
20 complex or as a single subunit under conditions as described herein; and the presence of histone acetyl (HAT) transferase activity.

"TAF subunits" refers to individual polypeptides that comprise the TAF complex activity. Such polypeptides are distinguished from any polypeptides previously known to be TBP binding proteins. Fungal TAF subunits may be
25 recombinant or purified from natural sources, and may include structural or functional TAF homologues as defined above.

A "fungal-specific epitope" of a fungal TAF subunit comprises a three-dimensional structural conformation presented by a folded or assembled TAF polypeptide that is not presented by the homologous mammalian sequence.

- 30 "Modulating transcription" means altering transcription, and includes increasing or decreasing the rate or level of transcription and changing the responsiveness of transcription to regulatory controls.

An "isolated" polypeptide or nucleic acid is defined as one that is

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5 unaccompanied by at least some of the material with which it is associated in its natural state. Generally, an isolated polypeptide constitutes at least about 1%, preferably at least about 10%, and more preferably at least about 50% by weight of the total protein in a given sample. Included in the polypeptide weight are alternative forms such as differentially glycosylated or phosphorylated or otherwise post-
10 translationally modified forms. An "isolated" nucleic acid sequence is present as other than a naturally occurring chromosome or transcript in its natural state and typically is removed from at least some of the proteins with which it is normally associated on a natural chromosome. A partially pure nucleotide sequence constitutes at least about 5%, preferably at least about 30%, and more preferably at least about 90% by weight
15 of total nucleic acid present in a given fraction.

Also encompassed by the invention are nucleic acids that are hybridizable to, or derived from, the TAF-145 sequences described above. In one embodiment, the invention relates to isolated nucleic acids capable of hybridizing with the TAF-145 sequences above or with their complements under the hybridization
20 conditions defined below.

-- Prehybridization treatment of the support (nitrocellulose filter or nylon membrane), to which is bound the nucleic acid capable of hybridizing with that of *C. albicans* TAF-145, at 65°C for 6 hours with a solution having the following composition: 4 x SSC, 10 x Denhardt (1X Denhardt is 1% Ficoll, 1%
25 polyvinylpyrrolidone, 1% BSA (bovine serum albumin); 1 x SSC consists of 0.15M of NaCl and 0.015M of sodium citrate, pH 7);

-- Replacement of the pre-hybridization solution in contact with the support by a buffer solution having the following composition: 4 x SSC, 1 x Denhardt, 25 mM NaPO₄, pH 7, 2 mM EDTA, 0.5% SDS, 100 µg/ml of sonicated
30 salmon sperm DNA containing a nucleic acid derived from the sequence of the TAF-145 as probe, in particular a radioactive probe, and previously denatured by a treatment at 100°C for 3 minutes;

-- Incubation for 12 hours at 65°C;

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5 -- Successive washings with the following solutions: (i) four washings with 2 x SSC, 1 x Denhardt, 0.5% SDS for 45 minutes at 65°C; (ii) two washings with 0.2 x SSC, 0.1 x SSC for 45 minutes at 65°C; and (iii) 0.1 x SSC, 0.1% SDS for 45 minutes at 65°C.

10 The invention also encompasses any nucleic acid exhibiting the property of hybridizing specifically with the above-described *C. albicans* TAF-145 under the conditions described above, but at 40°C, including successive washings in 2X SSC at 45°C for 15 minutes.

15 It will be understood that the conditions of hybridization defined above constitute preferred conditions for the hybridization, but are in no way limiting and may be modified without in any way affecting the properties of recognition and hybridization of the probes and nucleic acids mentioned above.

20 The salt conditions and temperature during the hybridization and the washing of the membranes can be modified in the sense of a greater or lesser stringency without the detection of the hybridization being affected. For example, it is possible to add formamide in order to lower the temperature during hybridization.

25 Nucleic acids that hybridize to the TAF-145 sequences of the invention may be of any length. In one embodiment, such polynucleotides are at least 25, preferably at least 100 and most preferably at least 200 nucleotides long. In another embodiment, the polynucleotide that hybridizes to the polynucleotide of the invention is of the same length as the polynucleotide of the invention.

TAF-145-Encoding Nucleic Acids and Polypeptides

30 The present invention encompasses nucleic acid sequences that encode TAF-145 from *C. albicans* and related *Candida* species. Methods for determining the relevant nucleic acid sequences are described in Example 1 below, and the deduced amino acid sequences of a TAF-145 gene, i.e. a gene encoding the 145 kDa TAF polypeptide (TAF-145) isolated from *C. albicans*, is shown in Figure 3. The present invention encompasses DNA and RNA sequences, and sense and antisense sequences.

5 TAF-encoding sequences according to the present invention may be modified by transitions, transversions, deletions, insertions, or other modifications such as alternative splicing. The invention also encompasses genomic TAF-145 sequences and TAF-145 gene flanking sequences, including TAF-145 regulatory sequences. Nucleic acid sequences encoding TAF-145 polypeptides may also be associated with
10 heterologous sequences, including promoters, enhancers, response elements, signal sequences, polyadenylation sequences, introns, 5'- and 3'- noncoding regions, and the like. Other useful heterologous sequences are known to those skilled in the art. Furthermore, the nucleic acids can be modified to alter stability, solubility, binding affinity and specificity. For example, TAF-145 encoding sequences can be selectively
15 methylated. The nucleic acid sequences of the present invention may also be modified with a label capable of providing a detectable signal, either directly or indirectly. Exemplary labels include radioisotopes, fluorescent molecules, biotin, and the like.

In general, nucleic acid manipulations according to the present
20 invention use methods that are well known in the art, as disclosed in e.g. *Molecular Cloning, A Laboratory Manual* (2nd Ed., Sambrook, Fritsch and Maniatis, Cold Spring Harbor), or *Current Protocols in Molecular Biology* (Eds. Ausubel, Brent, Kingston, More, Feidman, Smith and Stuhl, Greene Publ. Assoc., Wiley-Interscience, NY, NY, 1992).

25 The *C. albicans* TAF-145 of the present invention has not been disclosed or suggested in the prior art. Although there is some sequence homology between the *C. albicans* (SEQ ID NO:2) and *S. cerevisiae* (SEQ ID NO: 3) TAF-145 molecules in the central region of the protein (48%), there is a much lower sequence identity in the aminoterminal and carboxyterminal domains (19% and 29%,
30 respectively). In addition, there is a 74-amino acid sequence (residues 772 to 845) (SEQ ID NO:11) in *C. albicans* TAF-145, located between the central and C-terminal domains, which is not present in *S. cerevisiae*. Moreover, as shown below, *C. albicans* TAF-145 protein does not complement a *S. cerevisiae* TAF-145 temperature

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5 sensitive mutant protein when cells are grown at the restrictive temperature. Thus, the two molecules differ unpredictably with respect to both structure and function.

The invention also provides vectors comprising nucleic acids encoding *C. albicans* TAF-145 and analogs thereof. A large number of vectors, including plasmid and fungal vectors, have been described for expression in a variety of eukaryotic and prokaryotic hosts. Advantageously, vectors may also include a promoter and/or any other transcriptional regulatory sequence operably linked to the TAF-145 encoding portion. The encoded TAF-145 may be expressed by using any suitable vectors and host cells, using methods disclosed or cited herein or otherwise known to those skilled in the relevant art. The particular choice of vector/host is not critical to the invention.

Vectors will often include one or more replication systems for cloning or expression, one or more markers for selection in the host, e.g. antibiotic resistance, and one or more expression cassettes. The inserted TAF-145 coding sequences may be synthesized, isolated from natural sources, prepared as hybrids, etc. Ligation of the coding sequences to the transcriptional regulatory sequences may be achieved by known methods. Suitable host cells may be transformed/transfected/infected by any suitable method including electroporation, CaCl_2 mediated DNA uptake, fungal infection, microinjection, microprojectile, or other established methods.

Appropriate host cells include bacteria, archebacteria, fungi, especially yeast, and plant and animal cells, especially mammalian cells. Of particular interest are *E. coli*, *B. Subtilis*, *Saccharomyces cerevisiae*, *Pichia pastoris*, *Candida albicans*, other *Candida* species such as *C. tropicalis*, *C. parapsilosis*, *C. krusei*, and *C. glabrata*. *Aspergillus* species, SF9 cells, C129 cells, 293 cells, *Neurospora*, CHO cells, COS cells, HeLa cells, and immortalized mammalian myeloid and lymphoid cell lines. Preferred replication systems include M13, ColE1, SV40, baculovirus, lambda, adenovirus, and the like. A large number of transcription initiation and termination regulatory regions have been isolated and shown to be effective in the transcription and translation of heterologous proteins in the various hosts. Examples

5 of these regions, methods of isolation, manner of manipulation, etc. are known in the art. Under appropriate expression conditions, host cells can be used as a source of recombinantly produced TAF-145.

Nucleic acids encoding TAF-145 polypeptides may also be introduced into cells by recombination events. For example, such a sequence can be introduced
10 into a cell, and thereby effect homologous recombination at the site of an endogenous gene encoding TAF-145, an analog or pseudogene thereof, or a sequence with substantial identity to a TAF-145-encoding gene. Other recombination-based methods such as nonhomologous recombinations, deletion of endogenous gene by homologous recombination, especially in pluripotent cells, may also be used.

15 The present invention encompasses TAF complexes and subunits purified from wild-type and genetically altered strains of *C. albicans* or recombinantly produced in a non-native context. Yeast TAF complexes comprise about nine polypeptides, or closely related families of polypeptides. The complexes, and polypeptide components thereof, may be isolated by virtue of their affinity for fungal
20 or human TBP, by the use of chromatographic procedures that take advantage of physico-chemical characteristics of the complexes or of individual subunits, or by binding to TAF-specific antibodies. The isolated complexes may contain all, or only a subset, of the total known complement of TAF subunits. TAF multisubunit complexes may also be reconstituted and purified from translation products of subunit
25 genes, or from recombinantly produced TAF subunits. It is also contemplated that additional TAF subunit polypeptides will be identified using methods disclosed herein, and will be used in practicing the present invention.

In one embodiment, a baculovirus expression system permits the recombinant TAF-145 to be modified, processed and transported within a eukaryotic
30 system. In another embodiment, assembly of the TAF complex, or binding of preassembled TAF complexes to TBP, is performed in a reconstituted cell-free system using partially purified or substantially purified components. For example, TAF complexes, or components thereof, may be adsorbed to the surface of a microtiter

5 plate, and incubated with radiolabelled TBP protein. Functional binding of TBP to TAF complexes or components will result in the association of detectable radioactivity with the plate.

C. albicans TAF-145 according to the invention may be isolated from wild-type or mutant fungal cells, or from heterologous organisms or cells (including, but not limited to, bacteria, fungi, insect, plant, and mammalian cells) including
10 fungal cells into which a fungal-derived protein-coding sequence has been introduced and expressed. Furthermore, the TAF-145 sequence may be part of recombinant fusion proteins. Alternatively, TAF-145 polypeptides may be chemically synthesized by commercially available automated procedures, including, without limitation,
15 exclusive solid phase synthesis, partial solid phase methods, fragment condensation or classical solution synthesis.

Methods for polypeptide purification are well-known in the art, including, without limitation, preparative disc-gel electrophoresis, isoelectric focusing, HPLC, reversed-phase HPLC, gel filtration, ion exchange and partition
20 chromatography, and countercurrent distribution. For some purposes, it is preferable to produce TAF-145 in a recombinant system in which the fungal protein contains an additional sequence tag that facilitates purification, such as, but not limited to, a polyhistidine sequence. The polypeptide can then be purified from a crude lysate of the host cell by chromatography on an appropriate solid-phase matrix. Alternatively,
25 antibodies produced against TAF-145 or against peptides derived therefrom can be used as purification reagents. Other purification methods are possible.

The present invention also encompasses derivatives and homologues of *C. albicans* TAF-145. For some purposes, nucleic acid sequences encoding the peptides may be altered by substitutions, additions, or deletions that provide for
30 functionally equivalent molecules, i.e., function-conservative variants. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of similar properties, such as, for example, positively charged amino acids (arginine, lysine, and histidine); negatively charged amino acids (aspartate and

5 glutamate); polar neutral amino acids; and non-polar amino acids.

TAF-145 polypeptides can be modified by methods known in the art. For example, TAF-145 may be phosphorylated or dephosphorylated, glycosylated or deglycosylated, and the like. Especially useful are modifications that alter TAF-145 solubility, membrane transportability, stability, and binding specificity and affinity.

10 Some examples include fatty acid-acylation, proteolysis, and mutations in TBP interaction domains that stabilize binding.

TAF-145 of the present invention may also be modified with a label capable of providing a detectable signal, for example, at a heart muscle kinase labeling site, either directly or indirectly. Exemplary labels include radioisotopes, fluorescent compounds, etc. Such labeled TAFs thereof find use, for example, as probes in expression screening assays for proteins that interact with TAF, or in assays for TAF binding to TBP.

Identification of Functionally Important TAF Domains and Binding Partners

20 The polypeptides, protein complexes, and nucleic acids sequences of the present invention find use in the discovery, design, and development of pharmaceutically useful antifungal agents. The following embodiments of the present invention are directed towards elucidating epitopes and interactions of TAF-145 that can be selectively interfered with in a therapeutically beneficial manner.

25 In one embodiment, the known sequence of TAF-145 is used to design synthetic peptides comprising portions of the sequence. These peptides range from about 15 to about 50 amino acids in length. Peptides under 60 amino acids in length may be synthesized routinely using commercially available automated synthesizers. The peptides are then added to a cell-free assembly reaction containing, e.g.,
30 immobilized TAF complexes and soluble radiolabelled TBP. Determining which synthetic peptides inhibit some interaction of TAF-145 e.g. with other TAF subunits or with other factors, using routine experimentation, identifies different functional domains or epitopes of TAF-145. For example, a peptide fragment derived from

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5 TAF-145 that is found using the above-described method to inhibit the binding of
TBP to TAF-145 or to a TAF complex is likely to represent a region of TAF-145 that
interacts directly with TBP. In a similar manner, associational domains of different
TAF subunits that are involved in interactions among subunits, or between TAF
subunits or complexes and other transcriptional components, may be systematically
10 identified. These peptides may themselves constitute useful therapeutic reagents, or
may serve as the basis for design and formulation of pharmacologically active
compositions.

In another embodiment, important functional domains of TAFs are
identified using classical and reverse genetic methods that are well-known in the art.
15 For example, a nested set of deletion mutants can be prepared from the TAF-145
sequence. In this embodiment, progressively longer amino-terminal and carboxy-
terminal deletions can be engineered in the TAF-145 sequence. The resulting set of
mutant sequences can be individually expressed in a fungal strain under conditions in
which the wild-type version of the TAF is not expressed (see, e.g., Example 1 below,
20 in which *S. cerevisiae* was used as a host). By monitoring the function of each
mutant, it is possible to identify different regions of the TAF-145 polypeptide that are
critical for function i.e. functional domains or epitopes. Based on such studies, using
methods that are well-known in the art, it is possible to selectively introduce defined
mutations into different regions of the polypeptide and characterize the variant
25 protein's activity using a similar functional analysis.

An important aspect of the present invention is the selection of
functionally important domains or epitopes of *Candida* TAF-145 subunits that are
structurally and/or functionally distinct from their mammalian homologues. Such
domains are particularly useful as targets for antifungal drugs. In the case of TAF-
30 145, the *Candida* version differs in several important respects from its human
homologue, TAF-250. *Candida* TAF-145 is approximately half the size of human
TAF-250, and the homologous regions display an amino acid similarity and identity
of only 58% and 33%, respectively. *Candida* TAF-145 lacks the carboxy terminal

- 5 half of its human counterpart that contains the proposed "Bromo domains" and a region rich in acidic amino acid residues.

Identification of important structural and functional domains of TAFs according to the present invention enables the design and production of useful TAF-derived nucleic acid and peptide-based compounds. For example, fusion proteins may
10 be produced between an important TAF domain and e.g., an enzymatically active fragment of a DNA endonuclease. The resulting fusion protein, which can be produced in a fungal cell following introduction into the cell of the hybrid DNA operably linked to an expression vector, finds use in modulating TAF-dependent gene transcription. Other useful TAF fusion partners include sequences useful for
15 immobilization. For example, sequences derived from glutathione-S-transferase (GST) provide a binding site for immobilized glutathione, and sequences that form an epitope recognized by an available monoclonal antibody (e.g., 12CA5 monoclonal antibody) provide a binding site for the immobilized antibody.

In another example, particular serine, threonine, or tyrosine residues
20 in a TAF sequence may be identified as functionally important sites for phosphorylation of TAF. See e.g., methods disclosed in Roberts et al. (1991) *Science* 253, 1022-1026, and in Wegner et al. (1992) *Science* 256, 370-373. Phosphorylation of TAF subunits may be involved in modulating the transcription activation activity of Polymerase II transcribed genes. Identification of these residues will enable, first, the
25 radiolabelling of TAF subunits with γ -³²P-ATP. Furthermore, if phosphorylation of a particular residue is necessary for transcriptional activity, phosphorylation inhibitors may be designed to block activity.

The nucleic acids encoding TAF-145 may also be used to identify other nuclear factors that interact with TAF-145. In this embodiment, a yeast cDNA
30 library containing fusion genes of cDNA joined with DNA encoding the activation domain of a transcription factor (e.g., Gal4) is co-transfected with fusion genes encoding a portion of TAF and the DNA binding domain of a transcription factor. Clones encoding TAF binding proteins are able to complement the transcription factor

5 and are identified through transcription of a reporter gene. See, e.g., Fields et al. (1989) *Nature* 340: 245-246, and Chien et al., (1991) *Proc. Natl. Acad. Sci. USA* 88:9578-9582. It is contemplated that these additional binding partners for TAF will provide additional targets for antifungal drug therapy.

10 **Anti-TAF Antibodies**

The present invention encompasses antibodies that are specific for TAF-145 complexes or subunits identified as described above. The antibodies may be polyclonal or monoclonal, and may distinguish TAFs from other nuclear proteins, discriminate TAFs from those derived from different species, identify associational or other functional domains, and the like. Such antibodies are conveniently made using the methods and compositions disclosed in Harlow and Lane, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988, other references cited herein, as well as immunological and hybridoma technologies known to those in the art. Where natural or synthetic TAF-derived peptides are used to induce an TAF-specific immune response, the peptides may be conveniently coupled to an suitable carrier such as KLH and administered in a suitable adjuvant such as Freund's. Preferably, selected peptides are coupled to a lysine core carrier substantially according to the methods of Tam (1988) *Proc. Natl. Acad. Sci. USA* 85:5409-5413. The resulting antibodies may be modified to a monovalent form e.g. Fab, FAB', or FV. Anti-idiotypic antibodies, especially internal imaging anti-idiotypic antibodies, may also be prepared using known methods.

In one embodiment, purified *C. albicans* TAF-145 is used to immunize mice, after which their spleens are removed, and splenocytes used to form cell hybrids with myeloma cells and obtain clones of antibody-secreting cells according to conventional techniques. The resulting monoclonal antibodies are screened using *in vitro* assays such as those described above for the following activities: binding to TAF-145, inhibition of TAF-145 incorporation into multimeric TAF complexes, and inhibition of TAF-145 - TBP interaction.

5 In another embodiment, the entire TAF complex is used as an immunogen as above, and the resulting monoclonal antibodies are screened for their activity in inhibiting the *in vitro* assembly of any component of the TAF complex.

Anti-TAF antibodies may be used to identify and quantify TAF components, using immunoassays such as ELISA, EMIT, CEDIA, SLIFA, and the like. Anti-TAF antibodies may also be used to block the transcriptional function of, e.g., TAF-145 by inhibiting formation of complexes between TAF subunits or between assembled TAF complexes and other transcription components, or by immunodepleting cell extracts or transcription reactions of TAF components. In addition, these antibodies can be used to identify, isolate, and purify TAFs from different sources, and to perform subcellular and histochemical localization studies.

In one embodiment, polyclonal antibodies against the HAT domain of *C. albicans* TAF145 (aa 339-766) were generated by injection of purified recombinant protein into rabbits (Robert Sargeant, Ramona CA). The sera from these rabbits were then screened by ELISA (Figure 11) for their ability to recognize the recombinant CaTAF145 HAT domain (Example 5)

High-Throughput Drug Screening

The present invention encompasses the identification of agents useful in modulating fungal gene transcription, particularly the transcription of genes by RNA Polymerase II in a TAF-dependent manner. In a preferred embodiment, a high-throughput screening protocol is used to survey a large number of test compounds for their ability to interfere with TAF-dependent processes.

Test inhibitory compounds are screened from large libraries of synthetic or natural compounds. Numerous means are currently used for random and directed synthesis of saccharide, peptide, and nucleic acid based compounds. Synthetic compound libraries are commercially available from Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, NJ), Brandon Associates (Merrimack, NH), and Microsource (New Milford, CT). A rare chemical library is

5 available from Aldrich (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available from e.g. Pan Laboratories (Bothell, WA) or MycoSearch (NC), or are readily produceable. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and
10 biochemical means.

Useful inhibitory agents are identified using any suitable assay that employs TAF-145 or TAF-encoding nucleic acids. As examples, protein binding assays, nucleic acid binding assays and gel shift assays are useful approaches.

In one set of embodiments, TAF complexes or TAF-145 as provided
15 by the present invention are to be used in *in vitro* binding assays with either TBP alone or with a combination or subcombination of TBP and general transcription factors (GTFs).

For example, TAF complexes or TAF-145 may be immobilized on microtiter dishes using methods that are standard in the art. The plates are then
20 exposed to radiolabelled TBP e.g. [³²P]-TBP in the absence or presence of candidate compounds. Conversely, TBP may be immobilized, and incubated with radiolabelled TAF-145 or TAF complexes in the absence or presence of candidate compounds. Oligonucleotides comprising TBP target sequences may be used in conjunction with TBP and TAF. Postive "hit" compounds are those that inhibit TAF-TBP interaction.
25 In this case, incubation, washing, and radioactivity detection steps can be automated, allowing the screening of a large number of compounds, preferably at least about 1000 compounds per week.

In another embodiment, test compounds are screened to identify those that inhibit the histone acetyltransferase (HAT) activity of TAF-145 (see, e.g.,
30 Examples 2 and 3 below). Positive "hit" compounds are those that cause at least about 25%, preferably at least about 50%, and most preferably at least about 75%, inhibition of incorporation of [3H]-acetate into histones using the reaction conditions described in Example 2 below.

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5 In another embodiment, test compounds are screened to identify those that bind TAF-145, using the high-throughput screening methods described in U.S. Patent Nos. 5,585,277 and 5,679,582, in U.S.S.N. 08/547,889, and in PCT published application PCT/US96/19698. These methods may be used for identifying a ligand that binds the TAF 145 protein. According to these methods, a ligand, or a plurality
10 of ligands for TAF 145 target protein is identified by its ability to influence the extent of folding or the rate of folding or unfolding of the target protein. Experimental conditions are chosen so that the target protein is subjected to unfolding, whether reversible or irreversible. If the test ligand binds to the target protein under these conditions, the relative amount of folded:unfolded target protein or the rate of folding
15 or unfolding of the target protein in the presence of the test ligand will be different, i.e. higher or lower, than that observed in the absence of the test ligand. Thus, the method encompasses incubating TAF 145 in the presence and absence of a test ligand or ligands, under conditions in which (in the absence of ligand) the TAF 145 protein would partially or totally unfold. This is followed by analysis of the absolute or
20 relative amounts of folded vs. unfolded target protein or of the rate of folding or unfolding of the target protein.

An important feature of this method is that it will detect any compound that binds to any sequence or domain of the TAF 145, and not only to sequences or domains that are intimately involved in a biological activity or function. The binding
25 sequence, region, or domain may be present on the surface of the TAF 145 when it is in its folded state, or may be buried in the interior of the protein. Some binding sites may only become accessible to ligand binding when the protein is partially or totally unfolded.

Briefly, to carry out this method, the test ligand or ligands are
30 combined with the TAF 145, and the mixture is maintained under appropriate conditions and for a sufficient time to allow binding of the test ligand. Experimental conditions are determined empirically. When testing test ligands, incubation conditions are chosen so that most ligand:TAF 145 protein interactions would be

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5 expected to proceed to completion. The test ligand is present in molar excess relative to the TAF 145. The target protein can be in a soluble form, or, alternatively, can be bound to a solid phase matrix. The matrix may comprise without limitation beads, membrane filters, plastic surfaces, or other suitable solid supports.

10 In a preferred embodiment, binding of test ligand or ligands to TAF 145 is detected through the use of proteolysis. This assay is based on the increased susceptibility of unfolded, denatured polypeptides to protease digestion relative to that of folded proteins. In this case, the test ligand-TAF 145 protein combination, and a control combination lacking the test ligand, are treated with one or more proteases that act preferentially upon unfolded target protein. After an appropriate period of
15 incubation, the level of intact i.e. unproteolysed target protein is assessed using one of the methods described below e.g. gel electrophoresis and/or immunoassay.

There are two possible outcomes that indicate that the test ligand has bound the target protein. Either 1) a significantly higher, or 2) a significantly lower, absolute amount of intact or degraded protein may be observed in the presence of
20 ligand than in its absence.

Proteases useful in practicing the present invention include without limitation trypsin, chymotrypsin, V8 protease, elastase, carboxypeptidase, proteinase K, thermolysin, papain and subtilisin (all of which can be obtained from Sigma Chemical Co., St. Louis, MO). The most important criterion in selecting a protease or
25 proteases for use in practicing the present invention is that the protease(s) must be capable of digesting the TAF 145 protein under the chosen incubation conditions, and that this activity be preferentially directed towards the unfolded form of the protein. To avoid "false positive" results caused by test ligands that directly inhibit the protease, more than one protease, particularly proteases with different enzymatic
30 mechanisms of action, can be used simultaneously or in parallel assays. In addition, co-factors that are required for the activity of the protease(s) are provided in excess, to avoid false positive results due to test ligands that may sequester these factors.

In a typical embodiment of this method, purified TAF 145 protein is

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5 first taken up to a final concentration of 1-100 $\mu\text{g/ml}$ in a buffer containing 50 mM Tris-HCl, pH 7.5, 10% DMSO, 50 mM NaCl, 10% glycerol, and 1.0 mM DTT. Proteases, such as, for example, proteinase K or thermolysin (proteases with distinct mechanisms of action), are then added individually to a final concentration of 0.2-10.0 $\mu\text{g/ml}$. Parallel incubations are performed for different time periods ranging from 5
10 minutes to one hour, preferably 30 minutes, at 4°C, 15°C, 25°C, and 35°C. Reactions are terminated by addition of an appropriate protease inhibitor, such as, for example, phenylmethylsulfonyl chloride (PMSF) to a final concentration of 1mM (for serine proteases), ethylenediaminetetraacetic acid (EDTA) to a final concentration of 20 mM (for metalloproteases), or iodoacetamide (for cysteine proteases). The amount
15 of intact protein remaining in the reaction mixture at the end of the incubation period may then be assessed by any method, including without limitation polyacrylamide gel electrophoresis, ELISA, or binding to nitrocellulose filters. It will be understood that additional experiments employing a narrower range of temperatures can be performed to establish appropriate conditions. This protocol allows the selection of appropriate
20 conditions (e.g., protease concentration and digestion temperature) that result in digestion of approximately 70% of the target protein within a 30 minute incubation period, indicating that a significant degree of unfolding has occurred.

In another embodiment, the relative amount of folded and unfolded TAF 145 protein in the presence and absence of test ligand is assessed by measuring
25 the relative amount of the protein that binds to an appropriate surface. This method takes advantage of the increased propensity of unfolded proteins to adhere to surfaces, which is due to the increased surface area, and decrease in masking of hydrophobic residues, that results from unfolding. If a test ligand binds the TAF 145 (i.e., is a ligand), it may stabilize the folded form of the target protein and decrease its binding
30 to a solid surface. Alternatively, a ligand may stabilize the unfolded form of the protein and increase its binding to a solid surface.

Surfaces suitable for this purpose include without limitation microtiter plates constructed from a variety of treated or untreated plastics, plates treated for

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5 tissue culture or for high protein binding, nitrocellulose filters and PVDF filters.

In another embodiment, the extent to which folded and unfolded target protein are present in the test combination is assessed through the use of antibodies specific for either the unfolded state or the folded state of the protein i.e. denatured-specific ("DS"), or native-specific ("NS") antibodies, respectively. (Breyer, 1989, *J. Biol. Chem.*, 264 (5):13348-13354). Polyclonal or monoclonal antibodies are prepared as described above. The resulting antibodies are screened for preferential binding to the TAF 145 protein in its denatured state. These antibodies are used to screen for inhibitors of these interactions.

In another embodiment, molecular chaperones are used to assess the relative levels of folded and unfolded protein in a test combination. Chaperones encompass known proteins that bind unfolded proteins as part of their normal physiological function. In this embodiment, a test combination containing the test ligand and the TAF 145 is exposed to a solid support e.g. microtiter plate or other suitable surface coated with a molecular chaperone, under conditions appropriate for binding the TAF 145 with its ligand and binding of the molecular chaperone to unfolded target protein. The unfolded target protein in the solution will have a greater tendency to bind to the molecular chaperone-covered surface relative to the ligand-stabilized folded target protein. Thus, the ability of the test ligand to bind target protein can be determined by determining the amount of target protein remaining unbound, or the amount bound to the chaperone-coated surface. Alternatively, a competition assay for binding to molecular chaperones can be utilized.

Once conditions are established for high-throughput screening as described above, the protocol is repeated simultaneously with a large number of test ligands at concentrations ranging from 20 to 200 μ M. Observation of at least a two-fold increase or decrease in the extent of digestion of the target protein signifies a "hit" compound, i.e., a ligand that binds the target protein. Preferred conditions are those in which between 0.1% and 1% of test ligands are identified as "hit" compounds using this procedure.

5 In yet another embodiment, the test and control combinations described above can be contacted with a conformation-sensitive fluorescence probe, i.e., a probe that binds preferentially to the folded, unfolded, or molten globule state of the TAF 145 or whose fluorescence properties are in any way affected by the folding status of the TAF 145 protein. Once a particular test compound has been identified as described above, its activity is then confirmed by adding it to an *in vitro* transcription reaction, and measuring its effect on TAF-mediated activated transcription.

10 It is also contemplated that a useful agent may interfere with the function of TAF-145 but not inhibit TAF-TBP complex assembly. To screen for such compounds, other functional assays are used, such as, e.g., *in vitro* transcription reactions.

15 Finally, a test compound identified as described above is tested for two properties: its ability to inhibit fungal growth and its lack of effect on mammalian transcription. Fungal growth is measured by any method well-known in the art e.g. optical density of a liquid culture, or colony formation on agar. The lack of effect of a test compound on mammalian TAF-TBP interaction is tested by replacing yeast components with an analogous human *in vitro* transcription system as in, e.g., Manley et al. (1980) *Proc. Natl. Acad. Sci. USA* 77:3855-3859.

20 It will be understood that a compound that interferes with any aspect of TAF assembly or function is a likely candidate for an antifungal drug. Thus, in a manner similar to that described above for TBP-TAF-145 binding paradigm, binding assays can be routinely devised that measure the interaction of two or more TAF subunits with each other, or the interaction of one or more TAF subunits with other necessary transcription factors.

25 According to the present invention, useful agents may be found within numerous chemical classes, though typically they are organic compounds, and preferably small organic compounds. Small organic compounds have a molecular weight of more than 50 yet less than about 2,500 daltons, preferably less than about 750, more preferably less than about 250 daltons. Exemplary classes include

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5 peptides, saccharides, steroids, and the like. The compounds may be modified to enhance efficacy, stability, pharmaceutical compatibility, and the like. Structural identification of an agent may be used to identify, generate, or screen additional agents. For example, where peptide agents are identified, they may be modified in a variety of ways to enhance their stability, such as using an unnatural amino acid, such
10 as a D-amino acid, particularly D-alanine, by functionalizing the amino or carboxyl terminus, e.g., for the amino group, acylation or alkylation, and for the carboxyl group, esterification or amidification, or the like. Other methods of stabilization may include encapsulation, for example, in liposomes, etc.

15 **Therapeutic Applications**

For therapeutic uses such as the treatment of fungal infections in mammals, the compositions and agents disclosed herein may be administered by any convenient way, such as, e.g., parenterally, conveniently in a physiologically acceptable carrier, such as, e.g., phosphate buffered saline, saline, deionized water, or
20 the like. Typically, the compositions are added to a retained physiological fluid such as blood or synovial fluid. Alternatively, the compositions may comprise creams, ointments, lotions, or sprays for topical use. Generally, the amount administered will be empirically determined, typically in the range of about 10 to 1000 pg/kg of the recipient. For peptide agents, the concentration of will generally be in the range of
25 about 100 to 500 µg/ml in the dose administered. Other additives may be included, such as stabilizers, bactericides, etc. These additives will be present in conventional amounts.

Example 1: Cloning and Characterization of *Candida albicans* TAF-145

30 **A. *Degenerate oligonucleotide PCR***

A protein sequence alignment of the *Saccharomyces cerevisiae* (SEQ ID NO: 3) and TAF-145 counterparts from *Schizosaccharomyces pombe* (SEQ ID NO: 4), *Drosophila* (SEQ ID NO:5), and human (SEQ ID NO: 6) is shown in Figures 1A-1C.

5 Three highly conserved regions are boxed. Based on the sequence of these highly conserved regions, degenerate oligonucleotides (see Table 1 below) were designed and used to amplify fragments of DNA from *Candida albicans* strain SC5314 genomic DNA by polymerase chain reaction (PCR) (Figs. 2A, 2B and SEQ ID NO:12 - SEQ ID NO:15).

10 PCR reactions were performed with Taq DNA polymerase (Promega) in 25 μ l of buffer (provided by manufacturer) supplemented with 2 mM $MgCl_2$ and 200 μ M dNTP for a total of 30 cycles. Primers (shown in Table 1 below) were used at a concentration of 1.0 μ M with 100 ng of DNA and cycling was at 94°C (45 sec), 37°C (1.0 minute), and 72°C (1 minute) for four cycles followed by 94°C (45 sec), 40°C (1.0 minute), and 72°C (1 minute) for 26 cycles.

15 TABLE 1: Primers

Oligonucleotide	Sequence	Direction	Corresponding <i>S. cerevisiae</i> amino acids	SEQ ID NO:
TAF145p1F	5'-CC(A/T)GG(A/T)CC(A/T)AA(C/T) TCIA(A/G)(A/G)-3'	Forward	668-673	12
TAF145p2F	5'-GA(C/T)CC(A/T)AC(T/C/A)GG (A/T)TGTGG(A/T)GAAGG-3'	Forward	817-824	13
20 TAF145p3R	5'-CCTTTC(A/T)CCACA(A/T)CCAGT (A/T)GG(A/G)TC-3'	Reverse	817-824	14
TAF145p4R	5'-TT(A/G)TT(A/C/T)CA(C/T)CTIA (G/A)TG(T/A)CC-3'	Reverse	1045-1051	15

25 A major 700 bp fragment of DNA was amplified by use of the TAF145p1F and TAF145p3R primers. The amplified DNA was subcloned into the SrfI site of pCR-Script Amp SK(+) plasmid (Stratagene) and subjected to DNA sequencing (see below).

B. Cloning and sequence analysis of *C. albicans* TAF145

30 [³²P]-labeled DNA was generated by random hexamer priming (Boehringer Mannheim) of the cloned 700 bp *C. albicans* TAF-145 PCR product. The radiolabeled DNA was used to screen a *C. albicans* genomic DNA cosmid library by colony hybridization (Sambrook et al., 1989). The *C. albicans* genomic DNA

5 cosmid library was constructed using conventional procedures; the genomic DNA used to construct the library was purified from *C. albicans* strain SC5314 (Fonzi and Irwin, 1993) as described by Philippsen et al. (1991).

A 6.3 kb fragment of DNA containing the full length *C. albicans* TAF-145 (CaTAF145) gene was subcloned from a cosmid clone into pCT538, creating
10 pFL207 (see Table 2 below). All DNA manipulations were performed according to Sambrook et al. (1989). Figures 3A-3G display the entire nucleotide sequence of the *C. albicans* TAF145 gene (SEQ ID NO:1) and the predicted amino acid sequence (SEQ ID NO: 2) . *C. albicans* translates the standard leucine CUG codon as serine (Santos et al., 1996). Amino acids 980 and 1085 are shown as serines to reflect this
15 divergence from the standard genetic code.

Figures 4A-4D compare the amino amino acid sequences of *C. albicans* (SEQ ID NO:2) and *S. cerevisiae* (SEQ ID NO: 3) TAF145. While there is some sequence identity (48%) between *C. albicans* and *S. cerevisiae* in the middle portion of the protein, there is much lower sequence identity in the N-terminus and C-terminus portions (19% and 29%, respectively). The middle portion of the protein
20 contains a putative histone acetyltransferase domain (Mizzen et al., 1996). An additional difference between these two protein sequences is a 74-amino acid sequence (residues 772-845) in *C. albicans* TAF-145 (SEQ ID NO:11) , located between the central and C-terminus domains, which is not present in *S. cerevisiae*
25 TAF-145. Figures 5A-5C show a protein sequence alignment of the TAF-145 proteins from three yeast species: *C. albicans* (SEQ ID NO: 2) , *S. cerevisiae* (SEQ ID NO: 3) , and *S. pombe* (SEQ ID NO: 4) , and Figures 6A-6C display an alignment of the TAF-145-related proteins from *C. albicans* (SEQ ID NO:2) , *S. cerevisiae* (SEQ ID NO: 3) , *S. pombe* (SEQ ID NO: 4) , *Drosophila* (SEQ ID NO: 5) , and human
30 (SEQ ID NO: 6) . All sequence alignments were done with the Lasergene software package from DNASTAR Inc. The alignments illustrate the divergence of the aminoterminal and carboxyterminal domains of these proteins relative to the central domain.

5

C. Functional analysis of *Candida albicans* TAF-145

Two experiments were performed to determine if a genomic clone of *C. albicans* TAF-145 (CaTAF145) could substitute functionally for *S. cerevisiae* TAF-145 (ScTAF145). Plasmids and yeast strains used in these experiments are described in Tables 2 and 3, respectively. Yeast media, plasmids, and strains were prepared as described (Guthrie and Fink, 1991). Yeast transformations were done using a lithium acetate procedure (Schiestl and Gietz, 1989). Plasmid shuffle techniques were performed as described (Boeke et al., 1987) using 5-fluoro-orotic acid (5-FOA) as a selective agent against URA3 plasmids. All DNA manipulations used to generate the necessary plasmids were performed according to Sambrook et al. (1989).

15

TABLE 2

Plasmid	Description
pgTAF145	URA3 CEN ScTAF145
pTAF145 ^{ts-2}	HIS3 CEN ScTAF145 ^{ts-2}
pCT538	LEU2 2 μ
pFL112	LEU2 2 μ ScTAF145
pFL207	LEU2 2 μ CaTAF145
pCT3	URA3 CEN
pFL103	URA3 CEN ScTAF145
pFL115	URA3 CEN CaTAF145

20

25

TABLE 3

<i>S. cerevisiae</i> Strain	Genotype
YSW85	Mata lys2-801 ade2-101 leu2- Δ 1 his3- Δ 200 ura3- Δ 99 taf145::LEU2 [pgTAF145 (URA3 CEN ScTAF145)]
YSW101	Mata ade2-101 leu2-3,112 his3-11 ura3-1 trp1-1 taf145::hisG [pgTAF145 (URA3 CEN ScTAF145)]

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5	CTY507	Mata lys2-801 ade2-101 leu2- Δ 1 his3- Δ 200 ura3- Δ 99 taf145::LEU2 [pTAF145 ^{ts-2} (HIS3 CEN ScTAF145 ^{ts-2})]
	YFL021	Mata ade2-101 leu2-3,112 his3-11 ura3-1 trp1-1 taf145::hisG [pgTAF145 (URA3 CEN ScTAF145), pFL207 (LEU2 2 μ CaTAF145)]
	YFL022	Mata ade2-101 leu2-3,112 his3-11 ura3-1 trp1-1 taf145::hisG [pgTAF145 (URA3 CEN ScTAF145), pFL112 (LEU2 2 μ ScTAF145)]
	YFL023	Mata ade2-101 leu2-3,112 his3-11 ura3-1 trp1-1 taf145::hisG [pgTAF145 (URA3 CEN ScTAF145), pCT538 (LEU2 2 μ)]
	YFL024	Mata lys2-801 ade2-101 leu2- Δ 1 his3- Δ 200 ura3- Δ 99 taf145::LEU2 [pTAF145 ^{ts-2} (HIS3 CEN ScTAF145 ^{ts-2}), pFL115 (URA3 CEN CaTAF145)]
10	YFL025	Mata lys2-801 ade2-101 leu2- Δ 1 his3- Δ 200 ura3- Δ 99 taf145::LEU2 [pTAF145 ^{ts-2} (HIS3 CEN ScTAF145 ^{ts-2}), pFL103 (URA3 CEN ScTAF145)]
	YFL026	Mata lys2-801 ade2-101 leu2- Δ 1 his3- Δ 200 ura3- Δ 99 taf145::LEU2 [pTAF145 ^{ts-2} (HIS3 CEN ScTAF145 ^{ts-2}), pCT3 (URA3 CEN)]

One experiment took advantage of a temperature-sensitive (ts) mutant.

- 15 Plasmid shuffle techniques were used to create CTY507 (a *S. cerevisiae* strain with a temperature-sensitive allele of ScTAF145) from YSW85 and pTAF145^{ts-2} (Reese et al., 1994; Walker et al., 1996). CTY507 was then transformed with pCT3 ("empty" vector), pFL103 (ScTAF145), and pFL115 (CaTAF145) to generate *S. cerevisiae* strains YFL026, YFL025, and YFL024, respectively. Agar plates inoculated with
- 20 YFL024, YFL025, and YFL026 were placed at permissive (30°C) and restrictive (37°C) temperatures. At the permissive temperature, all three strains grew equally well (Figure 7A). In contrast, at the restrictive temperature, only cells transformed with wild-type *S. cerevisiae* TAF145 (YFL025) grew. Cells containing a genomic

- 5 clone of *C. albicans* TAF145 (YFL024) were unable to grow at the restrictive temperature.

A second experiment used plasmid shuffle techniques to determine if *C. albicans* TAF-145 could function in place of *S. cerevisiae* TAF-145. *S. cerevisiae* strain YSW101, containing a wild-type version of ScTAF145 on a URA3 plasmid, 10 was transformed with pCT538 (empty vector), pFL112 (ScTAF145), or pFL207 (CaTAF145) to generate *S. cerevisiae* strains YFL023, YFL022, and YFL021, respectively. Cells were placed on agar lacking leucine and selected for *Candida* TAF-145-encoding plasmids either in the absence or presence of 5-FOA (Figure 7B). All three strains grew equally well in the absence of 5-FOA. When the URA3- 15 containing ScTAF145 plasmid was selected against by the presence of 5-FOA, only those cells (YFL022) transformed by ScTAF145 were able to grow. In the absence of a functional *S. cerevisiae* TAF-145, cells containing a genomic clone of *C. albicans* TAF-145 were unable to grow.

Conditional mutant analysis and plasmid shuffle techniques in *S. 20 cerevisiae* show that a genomic clone of *C. albicans* TAF-145 was unable to substitute functionally *in vivo* for *S. cerevisiae* TAF145. In principle, the lack of complementation may be due to a lack of expression of mRNA and/or protein, or to a substitution of serines 980 and 1085 for leucine. A more likely explanation is that one or more of the protein surfaces required for interactions between yeast TAF-145 and 25 its partners may not be conserved between *S. cerevisiae* and *C. albicans*. While the middle portion of the two proteins share significant sequence similarity, the aminoterminal and carboxyterminal domains have diverged considerably (Figures 4A-4D).

D. Expression and purification of recombinant proteins

30 It has recently been reported that the *S. cerevisiae* TAF-145 protein and the human and *Drosophila* counterparts have histone acetyltransferase (HAT) activity associated with them (Mizzen et al., 1996). This activity has been mapped to the middle, most conserved, portion of ScTAF145 (amino acids 354-817) (SEQ ID

5 NO:8). Figures 8A and 8B display an alignment of the TAF-145 HAT domain from *C. albicans* (SEQ ID NO:7), *S. cerevisiae* (SEQ ID NO:8), *S. pombe* (SEQ ID NO:9), and human (SEQ ID NO: 10). The HAT domains derived from the three fungal species share approximately 50% amino acid sequence identity, while there is less than 25% amino acid sequence identity between the fungal and human TAF-145 HAT domains.

The portion of the gene encoding the putative HAT domains from *S. cerevisiae*, *C. albicans*, and human were PCR amplified and subcloned into pET19b (Novagen) to create pFL105, pFL107, and pFL108, respectively. Oligonucleotide sequences (SEQ ID NO:16 - SEQ ID NO:21) are listed in Table 4 below. Plasmids were transformed into *E. coli* BL21(DE3) for protein expression. Upon induction with IPTG, an amino-terminal His tagged protein was expressed.

TABLE 4

Oligo-nucleotide	Organism	Sequence	Direction	Corresponding amino acids and restriction sites created	SEQ ID NO:
pScHAT1	<i>S. cerevisiae</i>	5'-CCGCTCGAGAT GACACCCAACTTAAA GTTC-3'	Forward	amino acid 354 to... XhoI	16
pScHAT3	<i>S. cerevisiae</i>	5'-CGCGGATCCAGA GATTTTAGCTTAGAA TC-3'	Reverse	amino acid 787 BamHI	17
pCaHAT3	<i>C. albicans</i>	5'-GGAATTC CATATGCTTTTGCTC AACAATCCCTTGGAC -3'	Forward	amino acid 339 to... NdeI	18
pCaHAT4	<i>C. albicans</i>	5'-CGCGGATCCCT GCTCTGCTCACCGAA TAACAC-3'	Reverse	amino acid 766 BamHI	19
pHuHAT 6	Human	5'-GGAATTC CATATGAGCCTGGCA GGCTGGCTTCC TTCT-3'	Forward	amino acid 432 to... NdeI	20
pHuHAT 7	Human	5'-CCGCTCGAGTTC TGGAGCAAAAAGG ATTCTC-3'	Reverse	amino acid 912 XhoI	21

30 Restriction enzyme sites are underlined.

5 His-tagged HAT domain fusion proteins were purified on a nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography matrix (Qiagen) according to manufacturers instructions. Cells were lysed and proteins solubilized under denaturing conditions. Proteins were refolded on the Ni-NTA column and eluted with 0.5 M imidazole. Figure 9 shows the protein profile of extracts from
10 uninduced (U) and induced (I) cells following Ni-NTA chromatography. Addition of IPTG induced proteins of the expected size for the *S. cerevisiae* (Sc) and *C. albicans* (Ca) proteins (Figure 9).

Example 2: Histone acetyltransferase assays

15 Two assays for histone acetyltransferase (HAT) activity are described below. The first assay is an activity gel assay that permits direct association of polypeptides with acetyltransferase activity (Brownell and Allis, 1995). Purified HAT proteins are analyzed for HAT activity following electrophoresis in SDS/polyacrylamide gels containing calf thymus histones or bovine serum albumin.
20 Samples are dissolved in SDS/PAGE sample buffer, but not boiled, and then loaded onto standard SDS/polyacrylamide gels which have been modified so that protein substrates are dissolved in the resolving gel at 1 mg/ml prior to polymerization. Following electrophoresis, gels are washed for 1 hour at room temperature in buffer A (50 mM Tris-HCl pH8.0, 20%(v/v) isopropanol, 1 mM DTT, 1 mM PMSF, 0.1 mM
25 EDTA). Gels are then incubated in Buffer A containing 8 M urea for 1 hour and then overnight at 4°C in buffer A containing 0.04% Tween 40 (Sigma). The gels are then washed in buffer B (50 mM Tris-HCl pH8.0, 10%(v/v) glycerol, 1 mM DTT, 1 mM PMSF, 0.1 mM EDTA) prior to incubation with [³H]acetyl-CoA (10 µCi) for 30 minutes at 30°C. Finally, the labeled gels are washed with 5% trichloroacetic acid to
30 remove unbound radiolabel and fluorographed.

The second assay is a standard solution assay (Brownell and Allis, 1995). Enzyme samples are incubated at 30°C in a total volume of 50 µl of buffer B (50 mM Tris-HCl pH8.0, 10%(v/v) glycerol, 1 mM DTT, 1 mM PMSF, 0.1 mM

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- 5 EDTA) and 25 mg of calf thymus histones (Sigma). The reactions are initiated by the addition of [³H]acetyl-CoA (100 nCi; 6.1 Ci/mmol; ICN) to a final concentration of 0.5 μM and are terminated after 10 minutes by spotting the entire mixture onto Whatman P81 filters. [³H]-Acetate incorporation is determined by liquid scintillation and nonspecific counts are subtracted. This assay can be adapted for a high throughput
- 10 screen for the identification of compounds that inhibit the activity of fungal TAF145 acetyltransferase activity. A counterscreen with the human HAT domain protein is used to identify those inhibitors specific for the fungal proteins.

References for Examples 1 and 2

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40

Example 3: High-throughput Screening of Anti-TAF compounds

A. TAF binding

Corning ELISA strip wells (8 wells per strip) are coated with avidin (1.0 µg per well) by incubating avidin (200 µl of a 5 µg/ml stock) in coupling buffer

45 (per liter: 1.6g Na₂CO₃, 2.9 g, NaHCO₃, 0.9 g NaN₃) in the well for 12 h at 4°C. The

5 buffer is decanted, and nonspecific binding sites on the wells are blocked with 1% skim milk in phosphate-buffered saline (PBS) for 1 h at 37°C. Blocking buffer is discarded, and a yeast Pol II promoter-containing oligonucleotide (1 pmol/well) is added to the wells and incubated for 30 minutes at room temperature. The oligonucleotide is double-stranded and contains a biotin tag on the sense strand.

10 The oligonucleotide-containing solution is then removed, and the wells are washed with 1% milk in PBS. Yeast TBP is mixed with partially purified TAF that had been metabolically labelled with ³⁵S-methionine or purified TAF-145 similarly labelled, all in HEG buffer (0.1 M KCl, 25 mM HEPES pH 7.9, 0.5 mM EDTA, 20% glycerol, 0.01% LDAO, 0.1 M AEBSF, 0.1 M Na metabisulfite, 10 mM
15 β-mercaptoethanol) plus 200 ug/ml bovine serum albumin (BSA).

The protein mixture is then added to the prepared wells and incubated for 30 minutes at room temperature. Samples are then removed, and the wells are washed three times with the PBS/milk solution. Wells are separated and put into scintillation vials, scintillation cocktail is added, and samples are counted in a liquid
20 scintillation counter.

Binding of yeast TAF to the wells is found to be dependent on the presence of TBP, bound in turn to the Pol II promoter-containing oligonucleotide. Small molecules, whether purified or present in natural or synthetic mixtures, are introduced into the assay at concentrations ranging from about 20 to about 200 μM,
25 and appropriate solvent controls are also performed. Compounds that inhibit binding of TAF by more than about 30% are identified, and the inhibitory activity purified if not already available in pure form.

Compounds identified as described above are then tested for their ability to inhibit TBP-dependent transcription in a mammalian cell-free system.
30 Alternatively, human TBP is used in place of yeast TBP in the above-described assay for TAF binding to TBP. Thus, a subset of active compounds are identified that selectively interfere with yeast, and not mammalian, TAF-145 function, i.e., that are fungal-specific.

5 **B. HAT activity**

To each well of a microtiter dish is added 50 µl of a solution containing purified *C. albicans* TAF-145, buffer B (50 mM Tris-HCl pH8.0, 10%(v/v) glycerol, 1 mM DTT, 1 mM PMSF, 0.1 mM EDTA) and 25 µg of calf thymus histones (Sigma). Small molecules, whether purified or present in natural or synthetic mixtures, are introduced into the assay at concentrations ranging from about 20 to about 200 µM, and appropriate solvent controls are also performed.

The reactions are initiated by the addition of [³H]acetyl-CoA (100 nCi; 6.1 Ci/mmol; ICN) to a final concentration of 0.5 µM and are terminated after 10 minutes by spotting the entire mixture onto Whatman P81 filters. [³H]-Acetate incorporation is determined by liquid scintillation and nonspecific counts are subtracted. Compounds that inhibit [³H]-Acetate incorporation by at least about 25% are identified.

Compounds identified as described above are then tested for their ability to inhibit TBP-dependent transcription in a mammalian cell-free system. Alternatively, human TAF-145 is used in place of *Candida* TAF-145 in the above-described assay for HAT activity. Thus, a subset of active compounds are identified that selectively interfere with yeast, and not mammalian, TAF-145 function, i.e., that are fungal-specific.

Example 4: TAF Protein expression and purification for antibody production

Recombinant clones encoding for the HAT domain of the TAF protein, pFL 107 (*C. albicans* TAF 145 aa 339-766 in pET19b) [Figs. 8A, 8B and SEQ ID NO:7], pFL 110 (*S. cerevisiae* TAF 145 aa 354-835 in pET23a) [Figs. 8A, 8B and SEQ ID NO:8] and, pFL 123 (human TAF 250 aa 433-974 in pET23a) [Figs. 8A, 8B and SEQ ID NO:10] were each seeded into 10 ml of Luria Bertani (LB) broth supplemented with ampicillin (100 µg/ml) and grown overnight shaking at 37°C. The overnight cultures were seeded into 500 ml of LB/amp and grown shaking at 37°C. to mid-late log phase (O.D. of 0.6-0.8 at 600 nm). The cells were induced by the

- 5 addition of 0.4 M isopropyl β -D-thiogalactopyranoside (IPTG) (Sigma Chemical Co. St. Louis MO) and allowed to express the TAF proteins for 2 hours. The cells were harvested by centrifugation at 10,000 x g at 4°C. for 20 minutes and resuspended in Tris buffer (50mM Tris-HCL, pH. 8.0). The cells were lysed by sonication 3 x 3 minutes on ice using a Branson sonifier 250 (Branson Ultrasonics Corp., Danbury CT) and the soluble portion was separated from the insoluble portion by
- 10 centrifugation. The insoluble portion was solubilized by adding 3 ml of urea buffer (8 M urea) and incubated for 30 minutes to 2 hours at 37°C. The solubilized proteins were separated from cellular debris by centrifugation 10,000 x g for 20 minutes. SDS-PAGE sample buffer (0.5 M 2-mercapto-ethanol, 10% [wt./vol.] Sodium dodecyl
- 15 sulfate (SDS), 50% Glycerol, 0.5% Bromophenol blue) was added to the extracts and the samples boiled for 5 minutes.

The solubilized proteins from *C. albicans* (Ca), *S. cerevisiae* (Sc) and the human (Hu) TAF proteins were loaded onto a 12% SDS-polyacrylamide gel (3mm in thickness). The samples were electrophoresed until the bromphenol blue dye front

20 ran to the bottom of the gels. The proteins were visualized by staining the gels with Coomassie blue stain (10% acetic acid, .25% Coomassie Brilliant Blue) and destained overnight with destain (7% acetic acid, 30% methanol). Destain was

- 5 removed from the gels and the gels were washed 3 x for 10 minutes in deionized water.

For purification of the *C. albicans*, *S. cerevisiae*, and the human TAF proteins. The protein bands were cut from the gel and electroeluted using a Centrilutor device (Amicon Inc., Beverly MA) according to the manufacturer's instructions. The
10 purity of the electroeluted proteins was determined on a coomassie stained 12 % SDS-PAGE gel (Bio-Rad, Hercules CA) (Figure 10B). Each TAF protein band was discrete and free of other contaminating proteins.

The purified protein against the *C. albicans* TAF145 aa 339-766 [SEQ ID NO:7] was then injected into rabbits to produce polyclonal antibodies (see
15 Example 5).

Example 5: Production of antibodies against *C. albicans* TAF145 HAT domain

Polyclonal antibodies directed against a portion of the HAT domain of
20 *C. albicans* TAF145 (aa 339-766) [SEQ ID NO: 7] were generated by injection of purified recombinant protein into rabbits (Robert Sargeant, Ramona CA). Sera from these rabbits were tested for their ability to recognize recombinant CaTAF145 HAT domain in an ELISA assay according to the following method:

1. Varying amounts of CaTAF145 HAT domain were aliquotted in 50
25 mM Borate, pH8.5, were added to Immulon 4 plates, were incubated for 25°C for 1 hour, and the plates were then washed.

2. The plates were then blocked with 200 μ l of a 5% milk solution in a Tris buffer (TBST)

TBST = 10 mM Tris, pH 7.5
30 150 mM NaCl
0.5% Tween-20

The plates were incubated for 30 minutes at 25°C, and were then washed.

3. The washed plates were then incubated with rabbit antisera at multiple dilutions (1:500, 1:1000, 1:2000, 1:4000) in TBST(5% milk) for 1 hour at 25°C, and were then washed.

4. The plates were further incubated with a 1:1000 dilution of APGAR (Alkaline Phosphatase Conjugated Goat-anti-Rabbit polyclonal antibody Calbiochem) in TBST/5% milk. The secondary antibody was incubated for 1 hour at 25°C, and the plates were again washed.

5. The plates were then incubated with 2 mg/ml PNPP (para-nitrophenylphosphate) in a 1X diethanolamine buffer (Pierce) for approximately 20 minutes. Once color developed, the plates were read at OD 405 nm

15 The ELISA assay data for the four dilutions tested is graphically represented
in Figure 11.

Example 6: Expression of Human and *C. albicans* TAF proteins in Baculovirus

20 The full-length *C. albicans* TAF145 gene and a portion of the human TAF250 gene (aa 1-1218) [SEQ ID NO: 22] were PCR amplified and subcloned into pFastBac1 (Life Technologies Inc., Gaithersburg MD) to create pFL139 (*C. albicans*) and pFL149 (human), respectively. Oligonucleotide sequences (SEQ ID NO: 23- SEQ ID NO:28) are listed in the Table 5 below.

25

5

TABLE 5

Oligonucleotide	Organism	Sequence	Direction	Corresponding amino acids and restriction sites created	SEQ ID NO:
pCaHAT15	<i>C. albicans</i>	ACGCGTCGACATGC ATCATCATCATCAT CATATGGAGGATCT ACCCAGGGAT	Forward	amino acid 1 to ... 6xHis Tag and <i>SalI</i> site.	23
pCaHAT12	<i>C. albicans</i>	ATAGTTAGCGGCCG <u>C</u> ACACTGCTGGTGT CAACCAACAA	Reverse	amino acid 1161 NotI	24
pHuHAT11	Human	ACGCGTCGACATGC ATCATCATCATCAT CATATGGGACCCGG CTGCGATTG	Forward	amino acid 1 to... 6xHis Tag and <i>SalI</i> site.	25
pHuHAT12	Human	GTTGCT <u>CTG</u> CAGCT ATCATGCTATAATA AGC	Reverse	amino acid 894 PstI	26
pHuHAT13	Human	TGATAGCTGCAGAG CAACGACTGAAGG ATGC	Forward	amino acid 895 PstI	27
pHuHAT15	Human	CCGGTACCTTCCCG ATGTTGTTTCATCAA AAAG	Reverse	amino acid 1218 KpnI	28

All oligonucleotides are written in the 5' to 3' direction.

Restriction enzyme sites are underlined.

15

pFL149 was created in two steps: the 2.7 kb PCR product created by oligonucleotides pHuHAT11 and pHuHAT12 was inserted into pFastBac1 to create pFL141 followed by insertion of the 0.9 kb PCR product created by oligonucleotides pHuHAT13 and pHuHAT15. Bacmid DNA was generated by transforming pFL139 and pFL149 into *E. coli* DH10Bac and selecting on Luria Agar plates containing 50 mg/ml kanamycin, 7 mg/ml gentamicin, 10 mg/ml tetracycline, 100 mg/ml X-gal and, 40 mg/ml IPTG. White colonies were selected for the isolation of recombinant bacmid DNA. Bacmid DNA was isolated according to the BAC-To-BAC Baculovirus Expression Systems Instruction Manual (Life Technologies Inc., Gaithersburg MD).

25

The preparation of bacmid DNA and transfection of SF-9 cells was done using the Bac-to-Bac™ (Life Technologies Inc., Gaithersburg MD) expression system according to the instruction manual. 5 µl of bacmid mini-prep DNA

5 (recombinant and wild type) and 6 μ l of Cellfectin (Life Technologies Inc., Gaithersburg MD) reagent were each diluted into 100 μ l of grace's insect medium supplemented with L-glutamine (Invitrogen Corp., San Diego CA). Each solution was mixed by gently inverting several times. The bacmid DNA and cellfectin solutions were combined and allowed to incubate for 30 minutes at rt. 0.8 ml of
10 grace's minimal media was added to the complex and gently mixed by inverting.

9x10⁵ sf9 cells were seeded into each well (35mm) of a 6 well plate and allowed to attach for 1 hour at room temperature. The cells were washed 2 x with 2 ml of Grace's minimal media. After the final wash the cells were transfected with bacmid DNA-Cellfectin complex for 6 hours rocking at rt. The trasfection media was
15 removed and 2 ml of Ex-cell™ 420 serum-free insect media (JRH Biosciences, Lenexa KS) was added to each well and incubated for 72 hours at 27°C. After 72 hours the transfected cells were removed from the plates and harvested by low speed centrifugation. The supernatant fluid containing baculovirus was removed and stored at 4°C.

20 1 x 10⁶ High-Five cells, available from Invitrogen, Carlsbad, CA, were seeded in each well of a 6 well plate and allowed to attach for 1 hour at room temperature. The media was removed and the cells infected with 100 μ l of a 1:10 dilution of transfection baculovirus supernatant. The cells were rocked for 1 hour at rt. The infected cells were supplemented with 2 ml of Ex-Cell media and incubated for
25 72 hours at 27°C. After 72 hours the cells were collected by washing the wells several times with media and the cells pelleted by low speed centrifugation. The supernatant media was removed and the cells resuspended in phosphate-buffered saline (PBS), pH 7.5.

Coomassie stain and Western blot analysis was used to examine the
30 expression of recombinant TAF protein in Baculovirus (Figure 12). 5 μ l of 5x SDS-PAGE sample buffer was added to 10 μ l of the cell suspensions and the samples boiled for 5 minutes. Cellular debris was removed by centrifugation and the proteins (15 μ l) separated on 12% SDS-PAGE gels (Bio-Rad, Hercules CA). The gels were

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- 5 placed in a mini Trans-Blot apparatus (Bio-Rad, Hercules CA) and the proteins were electrophoretically transferred to nitrocellulose membranes. The membranes were blocked for 10 minutes with blocking buffer (5% skim milk, 20 mM Tris, 0.2 M NaCl, 0.1% Tween-20, pH 7.5). Rabbit polyclonal anti-*C. albicans* TAF 145 antibodies were used to probe the electroblotted infected high-five cell extracts. *C.*
- 10 *albicans* TAF 145 [SEQ ID NO: 2] and Human TAF 250 (aa 1-1218) [SEQ ID NO: 22] proteins were detected using goat anti-rabbit antibodies conjugated with HRP and ECL chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire England). Western Blot analysis illustrated that the Human (Figure 12; Lane 1) and the *C.*
- 15 *albicans* (Figure 12; Lane 2) HAT domains of the TAF protein were expressed in the Baculovirus system and the proteins were of the predicted size, 145 kDa.

Example 7: Deletion analysis of *C. albicans* TAF145

- 20 Deletion analysis of *C. albicans* TAF145 was used to establish if CaTAF145 is essential for cell viability. In wild-type *C. albicans*, CaTAF145 is encoded by two alleles (Figure 13B; lane 1).

- Homologous recombination was used to integrate two plasmids into the yeast genome. For a schematic diagram illustrating the homologous
- 25 recombination see Figure 13A. Each of the plasmids contain TAF145 interrupted by selectable markers as follows: ADE (pSIK11) and URA3 (pFL125).

Construction of plasmids pSIK11 and pFL125

pSIK11 was constructed in the following manner:

- 30 1) 460 bp CaTAF145 upstream region was PCR amplified with primers TAF145p32f (ACGCGTCGACATCCAAGTTCAAGTTGTCTG) [SEQ ID NO:29] and TAF145p33r (CGCGGATCCGCGCTGCAGTTTTTCACATCTTCTT CT TCTGCCA) [SEQ ID NO:30];

- 5 2) 518 bp CaTAF145 downstream region was PCR amplified with primers
TAF145p34f (AA~~AA~~ACTGCAGCGCGGATCC GCGT GCA GGTGA
CGTTATTGGA) [SEQ ID NO:31] and TAF145p35r (ATAGTTTAGCGGCCCGCC
TTGTGACAA GAAGTGACAC) [SEQ ID NO:32];
- 10 3) PCR products from steps 1 and 2 were used as templates and primers TAF145p32f
and TAF145p35r were used to generate a single piece of DNA by PCR that contains
the upstream and downstream region of CaTAF145;
- 15 4) the PCR product from step 3 was cloned into Sall-NotI sites of pBlueScript
(Stratagene, La Jolla, CA);
- 5) ADE2 gene was PCR amplified and inserted into PstI-BamHI sites of the plasmid
created in step 5.
- 20 pFL125 was constructed similarly to pSIK11 except that in step 5, hisG CaURA3
hisG (Fonzi and Irwin, 1993) was inserted into PstI-BamHI sites.
- Yeast medium was prepared as described (Guthrie and Fink, 1991).
Transformation of *C. albicans* was performed using a lithium acetate procedure
(Schiestl and Gietz, 1989). To create a single disruption of TAF145 in *C. albicans*
25 strain CAI8 (Fonzi and Irwin, 1993), cells were transformed with plasmid pSIK11
(Δ Cataf145::ADE2) digested with Sall/NotI and ADE+ prototrophs were selected.
Integration of the Δ Cataf145::ADE2 cassette at TAF145 was verified by Southern
Blotting and using 32 P- labeled pSIK11 as a probe (Figure 13B; Lane 2). These singly
disrupted strains (CSIK1) were selected for further analysis and then an attempt was
30 made to delete the second TAF145 allele.
- The singly disrupted strain CSIK1 (TAF145/ Δ Cataf145::ADE2) was
transformed with pFL125 (Δ Cataf145::URA3) digested with Sall/NotI and Ade+
Ura+ transformants were selected. As a control, to determine if pFL125

5 (Δ Cataf145::URA3) was capable of integrating into the yeast genome, CAI8 was transformed with Sall/NotI digested pFL125 (Δ Cataf145::URA3) and Ura+ transformants were selected. Analysis of a Ura+transformant is shown in Figure 13B, lane 3.

10 Ura+ Ade+ transformants were screened by Southern blot for the presence of both integrated cassettes (Figure 13B; Lanes 4-10). In 20 out of 20 transformants, the wildtype TAF145 band was still present, indicating that it was not possible to select for transformants with a deletion in the second copy of the TAF145 allele and that TAF145 is essential for cell viability. Figure 13B displays the results of Southern blot analysis for 7 of the 20 transformants. Each transformant selected
15 contained a deletion in only one copy of the TAF145 allele and only one selectable marker, either Ura or Ade but not both. This indicates that TAF145 is essential for cell viability.

References for Example 7

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30 Many variations of the present invention will suggest themselves to those skilled in the art in light of the above detailed disclosure. All such modifications are within the full extended scope of the appended claims. All patents and references mentioned in this application are hereby incorporated by reference in their entirety.